Cell Calcium xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene

Boglárka Zámbó[a], György Várady[a], Rita Padányi[b], Edit Szabó[a], Adrienn Németh[a], Tamás Langó[a], Ágnes Enyedi[b,c], Balázs Sarkadi[a,d,*]

[a] *Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar Tudosok krt. 2, Budapest, 1117, Hungary*
[b] *2nd Department of Pathology, Faculty of Medicine, Semmelweis University, Ulloi ut 26., Budapest, 1085, Hungary*
[c] *Molecular Oncology Research Group of the Hungarian Academy of Sciences and Semmelweis University, Ulloi ut 26., Budapest, 1085, Hungary*
[d] *MTA-SE Molecular Biophysics Research Group, Department of Biophysics and Radiation Biology, Semmelweis University, Tuzolto u. 37-43., Budapest, 1094, Hungary*

ARTICLE INFO

Article history:
Received 9 January 2017
Received in revised form 2 February 2017
Accepted 2 February 2017
Available online xxx

Keywords:
PMCA4b
ATP2B4
Haplotype
Promoter
Erythrocytes
Ca^{2+}

ABSTRACT

Plasma membrane Ca^{2+}-ATPases are key calcium exporter proteins in most tissues, and PMCA4b is the main calcium transporter in the human red blood cells (RBCs). In order to assess the expression level of PMCA4b, we have developed a flow cytometry and specific antibody binding method to quantitatively detect this protein in the erythrocyte membrane. Interestingly, we found several healthy volunteers showing significantly reduced expression of RBC-PMCA4b. Western blot analysis of isolated RBC membranes confirmed this observation, and indicated that there are no compensatory alterations in other PMCA isoforms. In addition, reduced PMCA4b levels correlated with a lower calcium extrusion capacity in these erythrocytes. When exploring the potential genetic background of the reduced PMCA4b levels, we found no missense mutations in the *ATP2B4* coding regions, while a formerly unrecognized minor haplotype in the predicted second promoter region closely correlated with lower erythrocyte PMCA4b protein levels. In recent GWA studies, SNPs in this *ATP2B4* haplotype have been linked to reduced mean corpuscular hemoglobin concentrations (MCHC), and to protection against malaria infection. Our data suggest that an altered regulation of gene expression is responsible for the reduced RBC-PMCA4b levels that is probably linked to the development of human disease-related phenotypes.

1. Introduction

Plasma membrane Ca^{2+}-ATPases (PMCAs) are key calcium exporters in most human cells, and these transporters are responsible for maintaining the low concentrations of cytoplasmic calcium, essential to calcium dependent signal transduction events. There are four isoforms of the PMCA proteins in the human body, and these isoforms and their several alternative transcript variants show tissue dependent occurrences [1]. PMCA1 and PMCA4 are ubiquitous; PMCA1 is essential for the survival of the embryos, while the lack of PMCA4, the main calcium efflux protein in sperms, results in male infertility [2,3]. The rapidly activated PMCA2 and PMCA3 transporters are predominantly present in the nervous system and muscle [4].

The *ATP2B4* gene encodes the PMCA4 protein with potentially 8 alternative transcript variants, resulting from the combinations of three splice sites [1]. The N-terminal splice site (A) results in the exclusion or inclusion of exon 8, and the formation of the z and x variants, respectively. The *in vivo* verification of the B splice site, potentially causing the exclusion of 38 amino acids from the protein, is controversial. The C-terminal alternative splice site (C) results in PMCA4 proteins with variable length in the C-terminal regulatory part. The PMCA4a protein has a shorter C-terminus, while the longer C-terminal region of the PMCA4b contains regulatory sites, including a calmodulin-binding region, a PKA phosphorylation site, a di-leucine motif and a PDZ-binding domain [5]. At low cytoplasmic calcium concentrations the active site of the PMCA4b protein is inhibited by the C-terminal tail, while Ca^{2+}-calmodulin binding causes tail detachment and the activation of the transporter. The PMCA4 variants show different tissue distribution,

* Corresponding author at: Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar Tudosok krt. 2, Budapest, 1117, Hungary.
E-mail address: sarkadi@biomembrane.hu (B. Sarkadi).

http://dx.doi.org/10.1016/j.ceca.2017.02.001
0143-4160/

and in the RBCs the main variant is PMCA4b, while low levels of the PMCA1 protein were also found to be present [1].

Recently we have developed a flow cytometry and antibody based method for the quantitative determination of protein levels in the RBC membrane. This method allows the rapid determination of numerous membrane proteins from a drop of blood (see [6]). Among several, potentially disease-related membrane proteins, we have also routinely determined the level of the relatively abundant RBC membrane PMCA4b protein, as a potential internal control, by using the PMCA4b variant specific JA3 antibody (see Methods). Interestingly, we found several healthy volunteers with significantly lower PMCA4b expression than the average values in the general population. The current report describes the exploration of the genetic background of this phenomenon, with a potential effect on health-related phenotypes.

2. Methods

2.1. RBC membrane protein studies

Blood samples obtained from 155 healthy volunteers were included in these measurements (this research was approved by the Hungarian ethics committee, ETTT). RBC membrane protein determinations were carried out according to our recently developed method [6–9]. In brief, we fixed and permeabilized the RBC membranes by using 1% formaldehyde solution, resulting in RBC "ghosts", and then equal number of cells were incubated with the PMCA-specific primary antibody (JA3, mouse monoclonal antibody, RRID: AB_628160) followed by a secondary, Alexa Fluor 488 labeled goat anti-mouse (H + L) antibody (Thermo Fisher/Life Technologies), in 96 well plates. We found that in these flow cytometry studies the PMCA4b specific JA3 monoclonal antibody gave the best RBC membrane labeling efficiency, while the JA9 mAb (recognizing both PMCA4 isoforms, RRID: AB_303296) and the 5F10 mAb (recognizing all PMCA variants, RRID: AB_303334) were less efficient. Cellular fluorescence was measured by FACSCanto II flow cytometer, equipped with plate loader.

Isolated human RBC membranes were prepared from 4 mL blood according to the protocol described in [10–12], and Western blots were carried out as described in [13–15]. Equal amounts of protein (determined by Lowry method) were loaded onto 7.5% acrylamide gels, electrophoresed, transferred onto PVDF membranes and immunostained. The following primary antibodies were used: mouse monoclonal antibodies anti-pan PMCA (5F10, RRID: AB_303334, dilution 1:100), anti-PMCA4 (JA9, RRID: AB_303296, dilution 1:250), and anti-PMCA4b (JA3, RRID: AB_628160, dilution 1:1500) and a rabbit polyclonal anti-PMCA1 antibody (NR1, dilution 1:500, [15]). Subsequently, HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch) were applied and detected by Pierce ECL Western Blotting Substrate (Thermo Scientific) and luminography. The ImageJ 1.50i software was used for densitometry analysis.

2.2. Calcium transport measurements in RBCs

The activity of the PMCA in RBCs was analysed by Fluo-4 fluorescence based calcium influx measurements according to Ref. [16], with slight modifications. In brief, intact RBCs were suspended in a high KCl buffer solution (10 mM HEPES, 70 mM NaCl, 80 mM KCl, 10 mM Inosine, 5 mM piruvate, 0.15 mM $MgCl_2$, 0.1 mM EGTA, pH7.4) and Fluo-4-AM (Molecular Probes/Invitrogen) was added in a final concentrations of 1 μM. RBCs were incubated for 1 h at 37 °C, and then 5 μL of the Fluo-4-loaded RBCs was added to 600 μL of a Ca^{2+} containing medium (10 mM HEPES, 70 mM NaCl, 80 mM KCl, 5 mM Inosine, 5 mM Glucose, 0.15 mM $MgCl_2$, 150 μM $CaCl_2$, pH 7.4) buffer on ice. The flow cytometry measurements were started by transferring the samples to room temperature and adding 0.8 μM final concentration of ionomycin (Molecular Probes/Invitrogen), then following RBC fluorescence by continuous sampling for 15 min. The area under the curve (AUC) was determined with integral calculation after normalization of the t_0 Fluo-4 fluorescence to zero (OriginPro 8 software).

2.3. Genetic analysis

Genomic DNA was purified from 300 μL of EDTA anticoagulated blood with Puregene Blood Kit (Qiagen). For sequencing the exons and exon-intron boundary regions of the *ATP2B4* gene, DNA was amplified by standard PCR method by using the respective primers (see Supplement Table S1), Sanger-sequencing was carried out by the Microsynth Company. TaqMan-based qPCR reactions for SNP detection were carried out in a StepOnePlus device (Applied Biosystems) with premade assay mixes (cat. 4351379) and master mix (cat. 4371353) from Thermo Fisher/Life Technologies. TaqMan probe specificity was verified with sequencing.

3. Results

3.1. PMCA4b protein levels in the RBC membrane

We quantified the level of PMCA4b expression in the RBC membrane of 155 healthy volunteers by flow cytometry (FACS) using the PMCA4b specific JA3 monoclonal antibody. In these experiments we found a number of volunteers with significantly lower than average PMCA expression levels (see Fig. 1A).

In order to confirm these data, the level of PMCA4b expression in the RBC membrane of selected individuals was also determined by Western blotting. This analysis showed similar PMCA expression patterns in all types of samples except that some particular volunteers had a significantly lower PMCA4b protein expression (Fig. 1B), in good correlation with the FACS results. We have also examined if there was a compensatory increase in the PMCA1 protein in the low RBC-PMCA4 membrane samples, but found no significant changes in this respect (see Fig. 1B).

3.2. Decreased expression of the PMCA4b causes reduced calcium extrusion in RBCs

In order to investigate if the reduced PMCA4b protein levels had any significant effect on the calcium homeostasis of the RBCs, we examined the calcium efflux properties of Fluo-4 loaded RBCs after stimulating calcium entry with ionomycin (see Methods and [16]). Fluo-4 loaded RBCs were placed into a medium with a relatively low concentration (150 μM) of Ca^{2+}, and then calcium entry was stimulated by the addition of 0.8 μM ionomycin. Using this method we were able to detect the intracellular calcium concentration in real time in live RBCs, and measured the response of the PMCA proteins to the elevated intracellular calcium concentration. Although, PMCAs are the only Ca^{2+} extrusion mechanisms in RBCs (PMCA4b being the most abundant isoform) that could actively eliminate Ca^{2+} from the cytosol [17], multiple ion channels and/or Ca^{2+} buffering proteins may potentially interfere with the assay. The results of three representative experiments in Fig. 1C show that RBCs expressing medium levels of PMCA4b are capable to compensate for the ionophore-induced calcium influx. In contrast, RBCs from volunteers with reduced PMCA4b levels showed reduced ability to compensate for the ionophore-induced rise in cellular calcium, most probably because of the reduced calcium efflux ability of these cells. These results suggest that changes in PMCA4b abundance by itself can effectively influence intracellular calcium levels in RBCs. The calculated area under the curve (AUC) showed

Please cite this article in press as: B. Zámbó, et al., Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene, Cell Calcium (2017), http://dx.doi.org/10.1016/j.ceca.2017.02.001

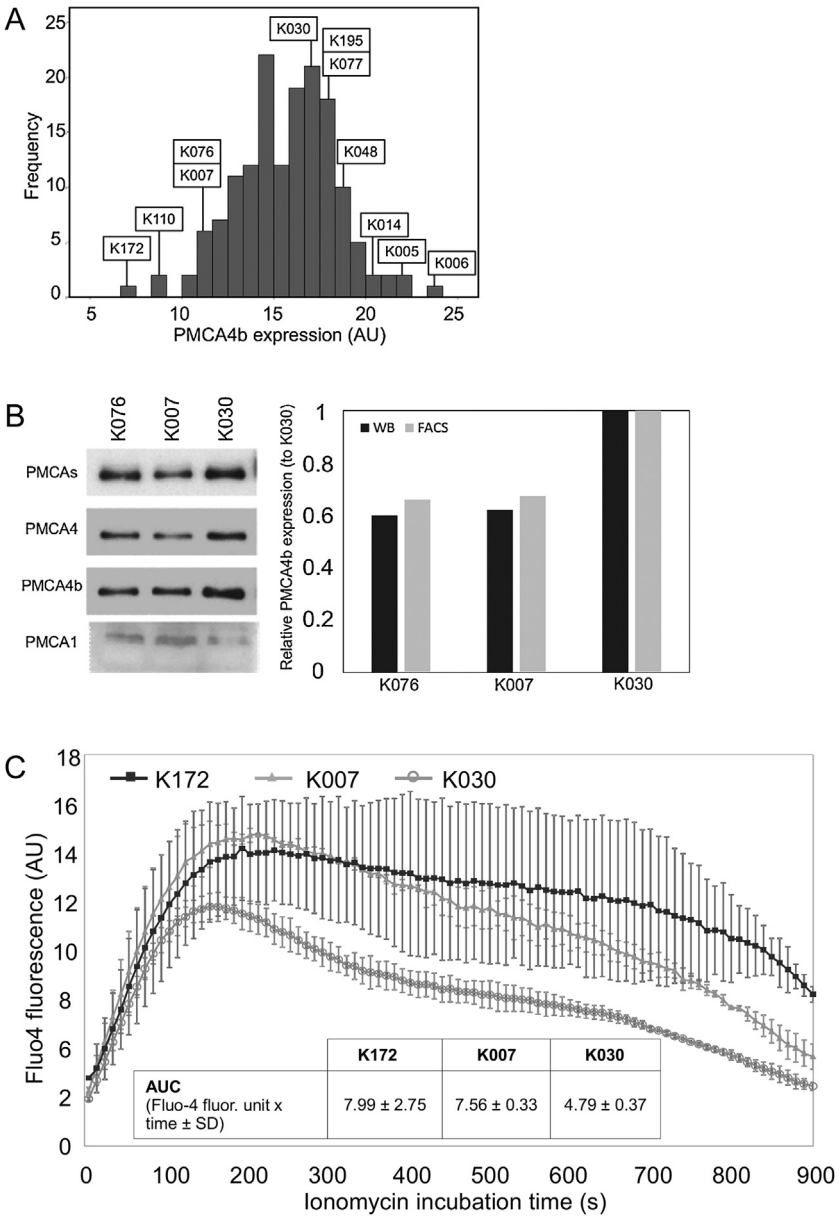

Fig. 1. Expression of the PMCA4b protein in the red blood cells of healthy volunteers.
(A) Distribution of PMCA4b protein levels as measured by quantitative flow cytometry (see Methods) in 155 healthy volunteers. Samples from individuals, which were further analysed are highlighted (K + donor number). (B) Reduced PMCA4b protein levels observed by quantitative flow cytometry correspond to lower plasma membrane expression of this protein as measured by Western blotting. Total PMCA levels (PMCAs) were assessed by using the 5F10 monoclonal antibody, recognizing all isoforms, while the levels of PMCA4 isoforms were assessed by isoform-specific antibodies (see Methods). (C) Reduced RBC-PMCA4b levels result in an impaired calcium pump activity in Fluo-4 loaded RBCs. Fluo-4 loaded RBCs were placed into a medium containing 150 μM of free calcium, and then calcium influx was induced by 0.8 μM ionomycin (see Methods). Under these conditions RBCs of most individuals were capable to down-regulate cellular calcium levels, while RBCs from donors with reduced PMCA4b levels showed impaired calcium extrusion. These experiments were performed altogether in seven healthy volunteers, three volunteers not carrying Haplotype 1, and three heterozygous and one homozygous volunteers for Haplotype 1. Independent samples from volunteers K030, K007 and K172 were measured three times, in each case with two parallels. Error bars represent the standard deviations of these three independent measurements with two parallels (AUC: area under the curve).

that RBCs with low PMCA4b abundance ($AUC_{K007} = 7.56 \pm 0.33$ and $AUC_{K172} = 7.99 \pm 2.75$) have an about 1.58–1.67 fold higher overall Ca^{2+} levels, compared to that in the control RBCs with average level of PMCA4b ($AUC_{K030} = 4.79 \pm 0.37$). Thus, a reduced PMCA4b level may directly impair calcium homeostasis in the RBCs of these otherwise healthy volunteers.

3.3. *A minor haplotype in the* ATP2B4 *gene correlates with decreased PMCA4b protein expression*

RBC membrane protein levels may be affected by direct genetic alterations or by various regulatory modulations (see [6,9]). According to our previous experience, strongly reduced RBC expression levels of the ABCG2 [7] and the ABCB6 [8] membrane proteins were connected to specific mutations and/or polymorphisms in the coding genes. Therefore, we analyzed the DNA extracts of the blood samples of the volunteers with low RBC-PMCA4b expression by Sanger sequencing of the *ATP2B4* gene, coding for this protein. The sequencing primers were selected to screen all exons and exon-intron boundaries of the *ATP2B4* gene.

When sequencing the *ATP2B4* genes of individuals with reduced RBC-PMCA4b protein levels (K007 and K076) we did not find any mutations or polymorphisms in the coding regions. In contrast, we

Please cite this article in press as: B. Zámbó, et al., Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene, Cell Calcium (2017), http://dx.doi.org/10.1016/j.ceca.2017.02.001

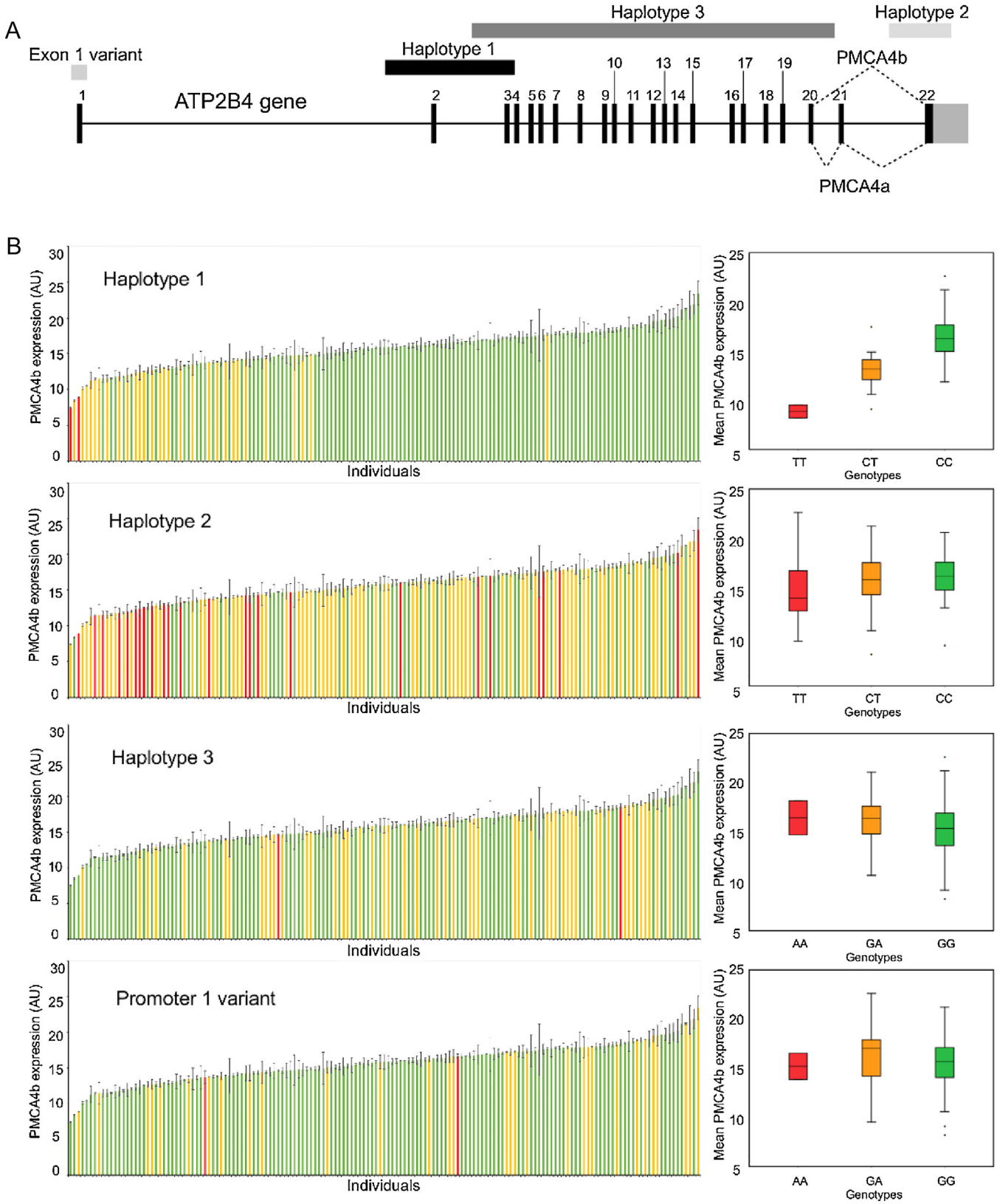

Fig. 2. Haplotypes in the ATP2B4 gene and their correlation with the level of PMCA4b expression in the red blood cells of healthy volunteers.)
(A) Haplotype/SNP localizations in the ATP2B4 gene. The two isoforms of the PMCA4 protein, 4a and 4b, which are different in their C-terminal regulatory regions, result from alternative transcript variants. (B) Left side: Relative expression levels of RBC-PMCA4b (155 samples) and the respective Haplotypes – lower PMCA4b protein levels in the RBCs are closely correlated with the presence of the minor variant of Haplotype 1.
Minor allele homozygotes: red, minor allele heterozygotes: yellow, major alleles: green. Right side: Mean values +/− SD values (at least two independent parallels), of the PMCA4b expression levels versus the ATP2B4 genotypes (Kruskal-Wallis tests).

Please cite this article in press as: B. Zámbó, et al., Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene, Cell Calcium (2017), http://dx.doi.org/10.1016/j.ceca.2017.02.001

found 7 SNPs in heterozygous forms in the non-coding regions of these samples, which were not present in samples with medium RBC-PMCA4b expression (K014, K030, K048, K077) (Fig. 2A). When further sequencing the DNA from individuals with very low RBC-PMCA4b expression levels (K110, K172) we found that the first 5 SNPs were present in a homozygous form, and the other two SNPs were either homozygous (K110) or heterozygous (K172). According to the LDLink data (NIH NCBI, [18]) all these five SNPs (rs1541252, rs1541253, rs377342347, rs1419114, rs2228445) and the other two SNPs (rs955865, rs955866) are inherited together in haplotypes (termed here by us as Haplotype 1 and Haplotype 2).

Haplotype 1 is in the 5′ region of the gene, between intron 1 to exon 3, and a proxy analysis of rs1419114 revealed that there are more than 50 short variants (including insertions and deletions) in this formerly unrecognized Haplotype, in a complete linkage disequilibrium ($R^2 > 0.9$, LDLink database). The minor allele frequency of the variants in this Haplotype 1 in Europe is about 0.11 (NCBI SNP database). In case of Haplotype 2, proxy analysis showed that about 9 variants are inherited together ($R^2 > 0.9$), but there are some other SNPs in the exon 20 and 3′UTR region of the gene which show slight linkage disequilibrium ($0.9 > R^2 > 0.2$) with these variants. The minor allele frequency of Haplotype 2 in Europe is about 0.44 (NCBI SNP database). Interestingly, Haplotypes 1 and 2 are in a weak linkage disequilibrium (Pearson correlation coefficient $R^2 = 0.1505$), and rs2228445 in Haplotype 1 shows high regulomeDB score (2b in LDLink), and relatively high R^2 coefficient in proxy search with Haplotype 2 ($R^2 = 0.1214$). These data suggest that SNP rs2228445 is a member of both haplotypes, and has a potential role in regulating membrane protein levels.

In addition, we have sequenced the coding region of the *ATP2B4* gene in individuals showing higher RBC-PMCA4b expressions than average (K195, K005, K006). Studying these sequences we observed another haplotype (rs3753036, Haplotype 3), which overlaps the first haplotype, and an SNP (rs4600103), which is a variant at the first exon of the gene. Haplotype 3 contains at least 36 more variants in linkage disequilibrium ($R^2 > 0.9$), with a minor allele frequency of 0.18 (NCBI database). The exon 1 variant shows weak ($R^2 < 0.8$) linkage disequilibrium with other SNPs close to its region in chromosome 1. The minor allele frequency of this exon 1 SNP is around 0.13 in the European population. It is important to note that none of these variants in the respective haplotypes cause any change in the amino acid sequence of the PMCA4 protein, most of them are in the intronic or UTR regions of the gene. The locations of the Haplotype 1–3 and the exon 1 SNP in the *ATP2B4* gene region are shown in Fig. 2A.

In the following experiments we analyzed the potential correlation of Haplotypes 1–3 with the RBC-PMCA4b levels. For this purpose we set up a TaqMan assay to screen a total of 155 blood samples, in which we already had the flow cytometry data for PMCA4b expression. We chose the rs1541252 (Haplotype 1), rs955866 (Haplotype 2), rs3753036 (Haplotype 3) as tagging SNPs to detect Haplotype 1, 2, and 3, respectively, and examined the SNP in exon 1 of the gene (rs4600103) as well.

The obtained results clearly show that the reduced RBC-PMCA4b protein levels correlate with the presence of the minor allele in Haplotype 1 (rs1541252 C > T, $p < 0.001$, Fig. 2B, Table 1). The second (rs955866 C > T) and third (rs3753036 G > A) haplotypes were only weakly related to the PMCA4b protein levels, probably because Haplotype 2 shows a weak linkage disequilibrium with Haplotype 1 (Pearson correlation coefficient $R^2 = 0.1505$), and Haplotype 3 overlaps Haplotype 1 (Fig. 2A). The SNP in exon 1 did not show any significant correlation with the PMCA4b protein levels. The presence of the minor Haplotype 1 in a heterozygous (CT) form reduced RBC-PMCA4b expression by about 25%, while in a homozygous (TT) form this minor Haplotype caused a 50% expression reduction (Table 2). The Haplotype variants showed a Hardy-Weinberg equilibrium (Haplotype 1: $p = 0.9900$, Haplotype 2: $p = 0.9113$, Haplotype 3: $p = 0.4163$, rs4600103: $p = 0.9999$, with Chi-square tests), the respective MAFs (minor allele frequencies) were q(T) = 0.1065, q(T) = 0.4161, q(A) = 0.1806, q(A) = 0.1129, in our sample set (155 samples of healthy individuals, Table 1). We successfully validated these results with a Sanger-sequencing of the affected *ATP2B4* regions in further 22 blood samples.

Table 1
Statistical analysis of the correlation of the haplotypes and the exon 1 variant of the *ATP2B4* gene with the RBC-PMCA4b expression levels.

n = 155 healthy subjects	p-value for correlation with PMCA expression (Kruskal-Wallis test)	Hardy-Weinberg eq. (Chi-square test)	q (MAF)
Haplotype 1	$p < 0.001$	0.9900	0.1065
Haplotype 2	$p = 0.012$ ($p < 0.05$)	0.9113	0.4161
Haplotype 3	$p = 0.040$ ($p < 0.05$)	0.4163	0.1806
Exon 1 variant	$p = 0.216$ (ns.)	0.9999	0.1129

Table 2
Mean RBC-PMCA4b expression levels of individuals carrying the *ATP2B4* genotypes.

	Genotype	Mean PMCA levels ± SD	n
Haplotype 1 (rs1541252)	TT	8.1771 ± 1.0404	2
	CT	12.9257 ± 1.7900	29
	CC	16.4842 ± 2.2529	124
Haplotype 2 (rs955866)	TT	14.5167 ± 3.1870	25
	CT	15.7618 ± 2.6973	79
	CC	16.2184 ± 2.3127	51
Haplotype 3 (rs3753036)	AA	16.6087 ± 2.7266	2
	GA	16.3905 ± 2.2263	52
	GG	15.3437 ± 2.8778	101
Exon 1 variant (rs4600103)	AA	15.1984 ± 2.1028	2
	GA	16.5170 ± 3.1788	31
	GG	15.5149 ± 2.5628	122
Total		15.7112 ± 2.7060	155

4. Discussion

In this study we found that PMCA4b expression, as measured by a flow cytometry method in the RBCs of healthy donors, showed a relatively constant average level, while in some donor RBCs the level of this transporter protein was much lower. We confirmed the lower PMCA4b expression levels by Western blotting in the respective isolated RBC membranes, and showed that the lower PMCA4b expression caused an impaired calcium efflux from the RBCs.

The localization and function of integral plasma membrane proteins, such as the PMCA4b protein, may be modulated by numerous factors, including transcriptional and translational regulation, protein trafficking or membrane lipid compositions. Since PMCAs are not glycosylated, and the plasma membrane is the only membrane compartment in RBCs, the most plausible explanation for the reduced RBC-PMCA4b expression is a transcriptional regulation. When examining the potential genetic background of the relatively low RBC-PMCA4b expression, we found no alterations in the coding regions that could result in amino acid changes, or altered splicing sequences in the *ATP2B4* gene, coding for this protein. Regarding the intronic and potential regulatory regions, our sequencing experiments showed a large number of variable SNPs, potentially involved in generating these changes in the protein expression level. We examined the correlation of three haplotypes in the *ATP2B4* gene with the protein expression, and found that the minor allele of a formerly unrecognized Haplotype 1 strongly correlates with low PMCA4b expression, namely heterozygotes show about 25%, while

Please cite this article in press as: B. Zámbó, et al., Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene, Cell Calcium (2017), http://dx.doi.org/10.1016/j.ceca.2017.02.001

homozygotes show about 50% of reduction of PMCA4b in the RBC membrane.

According to the Eukaryotic Promoter Database (EPDnew, [19]), there are two putative promoters in the *ATP2B4* gene. The first promoter is located at around the first exon, the second promoter is around the second exon, corresponding to Haplotype 1, shown here to affect PMCA4b expression. Our data suggest that SNPs in Haplotype 1 alter the binding of transcription factors which promote PMCA4b protein expression in myeloid blood cell differentiation, thus in RBC generation. Based on the PROMO (Alggen, [20]) database, the SNPs in Haplotype 1 may alter the binding sites for a large number of transcription factors, including GATA-1 and c-Myb, preferentially expressed in hematopoietic lineages. Further detailed studies are required to examine the role of the actual transcription factor binding sites in this region to regulate PMCA4 expression.

Haplotype 2 is located in the 3′ UTR of the *ATP2B4* gene and shows weak correlation ($R^2 = 0.1505$) with Haplotype 1, which may indicate an initial evolutionary assembly into a common haplotype. Haplotype 3 shows a partially overlapping location with Haplotype 1 (Fig. 2), and the minor allele of Haplotype 3 has a weak, but statistically significant effect to increase PMCA4b expression levels.

Haplotype 1 is located in a common region of the splice variants of the *ATP2B4* gene, coding both for PMCA4b and PMCA4a, thus Haplotype 1 may affect the expression regulation of both PMCA4 isotypes. Since it is difficult to obtain various human tissue samples for measuring quantitative PMCA4 expression levels, currently we do not know if the observed changes in the membrane PMCA levels are restricted to the RBCs or may affect the expression level of this protein in other tissues as well. Still, a potential correlation of the expression regulation of PMCA4 with disease phenotypes is a challenging question.

The PMCA4 protein is a key calcium transporter in various signaling and regulatory events. Specifically PMCA4 and PMCA1 were shown to be involved in osteoclast function; the altered expression of these PMCAs caused a decrease in the bone mass of menopausal women [21]. PMCA4a is the major calcium transporter protein in the human sperm and has an essential role in the motility of these cells, hence reduced PMCA4a might be involved in male sterility [2,3].

Several recent studies found potential associations between SNPs in the *ATP2B4* gene, and diseases or disease-related conditions, including reduced bone mass [21], increased incidence of schizophrenia [22], cardiac hypertrophy [23], red blood cell traits [24], and the incidence of malaria infection [25]. In GWAS studies the SNP rs7551442 in the *ATP2B4* gene was specifically linked to red blood cell MCHC (mean corpuscular hemoglobin concentration, Ref. [24]), while SNPs rs2365860, rs10900589, rs2365858 and rs4951074 were linked to malaria infection sensitivity [25]. These conditions may be linked phenotypes, e.g. impaired calcium pump activity in the myeloid progenitors may result in reduced RBC hemoglobin concentrations, altered cell shape and size, and in the mature RBCs may reduce Plasmodium infection. Plasmodium infection causes malaria, hence a reduction in Plasmodium infection may protect from, or ameliorate malaria. Interestingly, all these above SNPs found in the GWAS studies, are localized and thus inherited together, within a formerly unrecognized Haplotype 1 of *ATP2B4*.

Plasmodium malariae proliferates in the erythrocytes, where the parasite matures and multiplies within the parasitophorus vacuole (PV). This membrane vacuole is an invagination of the RBC plasma membrane, which contains the proteins of the host erythrocyte, including PMCA4b. Recent studies [26,27] suggest that the parasite growth needs a sufficiently high calcium level in the PVs, and while the $[Ca^{2+}]$ in the RBC cytoplasm is very low, it is much higher ($\sim$40 μM) within the PVs. Since PMCA4b is present in an inverted situation in the PV membrane, it pumps Ca^{2+} into the PV, thus generating the high Ca^{2+} microenvironment for the parasite. Therefore, individuals carrying the *ATP2B4* haplotype with a reduced level of PMCA4b may be protected against malaria due to a lower calcium level in the PV, causing reduced proliferation of the parasite.

Here we document a major reduction of this calcium transporter protein in the RBCs of individuals carrying the minor variant of Haplotype 1. Our studies thus establish a strong correlation between the gene-regulation based changes in membrane PMCA levels and the development of human disease-related phenotypes. Based on this study, we further emphasize the advantages of the direct measurements of RBC membrane proteins in discovering genetic regulatory effects, and their potential in future personalized diagnostic approaches.

Competing interests

We have no conflict of interest to report.

Author contributions

Contributions: B.Z. designed and performed the experiments, analyzed the data; Gy.V. developed the antibody based FACS method, Gy.V., E.Sz., A.N. and B.Z. performed and analysed the antibody based FACS measurement; R.P. and Á.E. designed the Western-blot and R.P. performed the Western-blot measurements; T.L. and B.Z. performed the isolation of RBC membrane; Á.E. and B.S. designed the concept of the study, analyzed the data; B.Z. and B.S. wrote the manuscript. All authors reviewed the manuscript.

Acknowledgements

We greatly appreciate the advice and help of Michael Dean, NIH, USA, in analyzing the SNP databases, and the technical assistance by Krisztina Lór. This study has been supported by grants from NKFIH (K115375, FIEK16 to BS; and ANN110922, K119223 to AE).

References

[1] E.E. Strehler, D.A. Zacharias, Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps, Physiol. Rev. 81 (2001) 21–50, http://physrev.physiology.org/content/81/1/21.abstract.
[2] G.W. Okunade, M.L. Miller, G.J. Pyne, R.L. Sutliff, K.T. O'Connor, J.C. Neumann, A. Andringa, D.A. Miller, V. Prasad, T. Doetschman, R.J. Paul, G.E. Shull, Targeted ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4, J. Biol. Chem. 279 (2004) 33742–33750, http://dx.doi.org/10.1074/jbc.M404628200.
[3] K. Schuh, E.J. Cartwright, E. Jankevics, K. Bundschu, J. Liebermann, J.C. Williams, A.L. Armesilla, M. Emerson, D. Oceandy, K.-P. Knobeloch, L. Neyses, Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility, J. Biol. Chem. 279 (2004) 28220–28226, http://dx.doi.org/10.1074/jbc.M312599200.
[4] W.L. Stahl, T.J. Eakin, J.W.M. Owens Jr., J.F. Breininger, P.E. Filuk, W.R. Anderson, Plasma membrane Ca2+ ATPase isoforms: distribution of mRNAs in rat brain by in situ hybridization, Mol. Brain Res. 16 (1992) 223–231, http://dx.doi.org/10.1016/0169-328X(92)90229-5.
[5] R. Padányi, K. Pászty, L. Hegedűs, K. Varga, B. Papp, J.T. Penniston, Á. Enyedi, Multifaceted plasma membrane Ca2+ pumps: from structure to intracellular Ca2+ handling and cancer, Biochim. Biophys. Acta Mol. Cell Res. 1863 (2016) 1351–1363, http://dx.doi.org/10.1016/j.bbamcr.2015.12.011.
[6] B. Sarkadi, G. Varady, I. Kasza, Quantitative biomarkers in the erythrocyte membrane., Google Pat. EP2839293A2. (2013).
[7] I. Kasza, G. Várady, H. Andrikovics, M. Koszarska, A. Tordai, G.L. Scheffer, A. Németh, G. Szakács, B. Sarkadi, Expression levels of the ABCG2 multidrug transporter in human erythrocytes correspond to pharmacologically relevant genetic variations, PLoS One 7 (2012) e48423, 10.1371%2Fjournal.pone.0048423.
[8] M. Koszarska, N. Kucsma, K. Kiss, G. Varady, M. Gera, G. Antalffy, H. Andrikovics, A. Tordai, M. Studzian, D. Strapagiel, L. Pulaski, Y. Tani, B. Sarkadi, G. Szakacs, Screening the expression of ABCB6 in erythrocytes reveals an unexpectedly high frequency of Lan mutations in healthy individuals, PLoS One 9 (2014) e111590, 10.1371%2Fjournal.pone.0111590.
[9] G. Várady, J. Cserepes, A. Németh, E. Szabó, B. Sarkadi, Cell surface membrane proteins as personalized biomarkers: where we stand and where we are

headed, Biomark. Med. 7 (2013) 803–819, http://dx.doi.org/10.2217/bmm.13.90.
[10] T. Hegedűs, P.M. Chaubey, G. Várady, E. Szabó, H. Sarankó, L. Hofstetter, B. Roschitzki, B. Stieger, B. Sarkadi, Inconsistencies in the red blood cell membrane proteome analysis: generation of a database for research and diagnostic applications, Database bav056 (2015), http://dx.doi.org/10.1093/database/bav056.
[11] H.J. Schatzmann, G.L. Rossi, (Ca2+ + Mg2+)-activated membrane ATPases in human red cells and their possible relations to cation transport, Biochim. Biophys. Acta Biomembr. 241 (1971) 379–392, http://dx.doi.org/10.1016/0005-2736(71)90037-X.
[12] H.U. Wolf, Studies on a Ca2+-dependent ATPase of human erythrocyte membranes, Biochim. Biophys. Acta Biomembr. 266 (1972) 361–375, http://dx.doi.org/10.1016/0005-2736(72)90094-6.
[13] R. Padányi, K. Pászty, E.E. Strehler, Á. Enyedi, PSD-95 mediates membrane clustering of the human plasma membrane Ca2+ pump isoform 4b, Biochim. Biophys. Acta Mol. Cell Res. 2009 (1793) 1023–1032, http://dx.doi.org/10.1016/j.bbamcr.2008.11.007.
[14] A.J. Caride, A.G. Filoteo, A. Enyedi, A.K. Verma, J.T. Penniston, Detection of isoform 4 of the plasma membrane calcium pump in human tissues by using isoform-specific monoclonal antibodies, Biochem. J. 316 (1996) 353–359, http://www.biochemj.org/content/316/1/353.abstract.
[15] A.G. Filoteo, N.L. Elwess, A. Enyedi, A. Caride, H.H. Aung, J.T. Penniston, Plasma membrane Ca2+ pump in rat brain: patterns of alternative splices seen by isoform-specific antibodies, J. Biol. Chem. 272 (1997) 23741–23747, http://dx.doi.org/10.1074/jbc.272.38.23741.
[16] K. de Jong, F.A. Kuypers, Flow cytometric determination of PMCA-mediated Ca2+-extrusion in individual red blood cells, Cytom. Part A 71A (2007) 693–699, http://dx.doi.org/10.1002/cyto.a.20429.
[17] A. Bogdanova, A. Makhro, J. Wang, P. Lipp, L. Kaestner, Calcium in red blood cells – a perilous balance, Int. J. Mol. Sci. 14 (2013) 9848–9872, http://dx.doi.org/10.3390/ijms14059848.
[18] M.J. Machiela, S.J. Chanock, LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants, Bioinforma (2015), http://dx.doi.org/10.1093/bioinformatics/btv402.
[19] R. Dreos, G. Ambrosini, R.C. Périer, P. Bucher, The eukaryotic promoter database: expansion of EPDnew and new promoter analysis tools, Nucleic Acids Res. 43 (2015) D92–D96, http://dx.doi.org/10.1093/nar/gku1111.
[20] X. Messeguer, R. Escudero, D. Farré, O. Núñez, J. Martınez, M.M. Albà, PROMO: detection of known transcription regulatory elements using species-tailored searches, Bioinforma 18 (2002) 333–334, http://dx.doi.org/10.1093/bioinformatics/18.2.333.
[21] H.J. Kim, V. Prasad, S.-W. Hyung, Z.H. Lee, S.-W. Lee, A. Bhargava, D. Pearce, Y. Lee, H.-H. Kim, Plasma membrane calcium ATP-ase regulates bone mass by fine-tuning osteoclast differentiation and survival, J. Cell Biol. 199 (2012) 1145–1158, http://jcb.rupress.org/content/199/7/1145.abstract.
[22] D. Martins-de-Souza, W.F. Gattaz, A. Schmitt, C. Rewerts, S. Marangoni, J.C. Novello, G. Maccarrone, C.W. Turck, E. Dias-Neto, Alterations in oligodendrocyte proteins, calcium homeostasis and new potential markers in schizophrenia anterior temporal lobe are revealed by shotgun proteome analysis, J. Neural Transm. 116 (2009) 275–289, http://dx.doi.org/10.1007/s00702-008-0156-y.
[23] X. Wu, B. Chang, N.S. Blair, M. Sargent, A.J. York, J. Robbins, G.E. Shull, J.D. Molkentin, Plasma membrane Ca2+-ATPase isoform 4 antagonizes cardiac hypertrophy in association with calcineurin inhibition in rodents, J. Clin. Invest. 119 (2009) 976–985, http://dx.doi.org/10.1172/JCI36693.
[24] J. Li, J.T. Glessner, H. Zhang, C. Hou, Z. Wei, J.P. Bradfield, F.D. Mentch, Y. Guo, C. Kim, Q. Xia, R.M. Chiavacci, K.A. Thomas, H. Qiu, S.F.A. Grant, S.L. Furth, H. Hakonarson, P.M.A. Sleiman, GWAS of blood cell traits identifies novel associated loci and epistatic interactions in Caucasian and African American children, Hum. Mol. Genet. (2012), http://dx.doi.org/10.1093/hmg/dds534.
[25] C. Timmann, T. Thye, M. Vens, J. Evans, J. May, C. Ehmen, J. Sievertsen, B. Muntau, G. Ruge, W. Loag, D. Ansong, S. Antwi, E. Asafo-Adjei, S.B. Nguah, K.O. Kwakye, A.O.Y. Akoto, J. Sylverken, M. Brendel, K. Schuldt, C. Loley, A. Franke, C.G. Meyer, T. Agbenyega, A. Ziegler, R.D. Horstmann, Genome-wide association study indicates two novel resistance loci for severe malaria, Nature 489 (2012) 443–446, http://dx.doi.org/10.1038/nature11334.
[26] T. Tiffert, H.M. Staines, J.C. Ellory, V.L. Lew, Functional state of the plasma membrane Ca2+ pump in Plasmodium falciparum-infected human red blood cells, J. Physiol. 525 (2000) 125–134, http://dx.doi.org/10.1111/j.1469-7793.2000.00125.x.
[27] M.L. Gazarini, A.P. Thomas, T. Pozzan, C.R.S. Garcia, Calcium signaling in a low calcium environment: how the intracellular malaria parasite solves the problem, J. Cell Biol. 161 (2003) 103–110, http://jcb.rupress.org/content/161/1/103.abstract.

Please cite this article in press as: B. Zámbó, et al., Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the *ATP2B4* gene, Cell Calcium (2017), http://dx.doi.org/10.1016/j.ceca.2017.02.001